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(54) Title: MULTIDOMAIN HEMATOPOIESIS STIMULATORS

(57) Abstract

Provided are novel fusion proteins, IL3-X or X-IL3, wherein X is a lymphokine fused to IL3.

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MULTIDOMAIN HEMATOPOIESIS STIMULATORS

The present invention relates to fusion molecules characterized by the presence of interleukin 3 [IL3] fused to a second lymphokine either with or without a linker sequence. The fusion molecule may be characterized by having the usual activity of both of the peptides forming the fusion molecule or it may be further characterized by having a biological or physiological activity greater than simply the additive function of the presence of IL3 or X. The fusion molecule may also unexpectedly provide an enhanced effect on the activity of each of the fusion protein or an activity different from that expected by the presence of IL3 or X.

The novel IL3-X or X-IL3 fusion protein provided by the present invention is a homogeneous protein substantially free of association with other mammalian proteinaceous materials. The entity X in the above formulae represents a lymphokine, the DNA coding sequence of which is fused in frame with the DNA coding region of IL3, either directly or through a linker. By "fused in frame" is meant that there is no translational terminator between the reading frames of the IL3 and X proteins. As used herein, the term "directly" defines fusion of the DNA sequences encoding X and IL3 without a peptide linker. The X entity may be fused to either the 5' or 3' end of the IL3 cDNA molecule.

The DNA and protein sequences of the IL3 molecule are published and may be constructed by a variety of techniques now standard in the art. See, e.g., PCT publication WO 88/00598, published January 28, 1988.

The lymphokines included within the definition of X are selected from the group consisting of GMCSF, GCSF, erythropoietin, IL1, IL2, IL3, IL4, IL6, IL7, IL9, IL11 or B cell stimulatory factor. Presently preferred X lymphokines for inclusion are selected from the group consisting of IL3, IL6, IL7, IL9, IL11 and GCSF. Additionally, this invention encompasses the use of modified X molecules or mutated or

modified DNA sequences encoding these X molecules. Polypeptide and DNA s quences (native and modified) for these lymphokines are published in the art, as are methods for obtaining expression thereof through recombinant or chemical synthetic techniques.

Fusing of the IL3 sequence to the sequence of X may be accomplished by use of intermediate vectors as described in the examples below. Alternatively, the IL3 sequence can be inserted directly into a vector which contains the X protein coding region, or vice versa. Techniques for cloning DNA sequences in phages or plasmids are known to those of skill in the art. Thus, the gene for the fusion protein IL3-GCSF, for example, is constructed in a vector comprising DNA sequences encoding the two domains fused in frame with one another and operatively linked either directly or through a peptide linker to a regulatory region capable of controlling expression of the genes in the appropriate host cell. The fusion may be performed by conventional techniques. [See e.g., Sambrook et al, "Molecular Cloning. A Laboratory Manual", Cold Spring Harbor Laboratory (1989)].

Linkers and adapters can be used for joining the IL3 and X sequences, as well as replacing lost sequences, where a restriction site employed was internal to the region of interest. The linkers joining the two molecules are preferably designed to allow the IL3 and X proteins to fold and act independently of one another. The sequence of one exemplary linker used in the present examples was based on a sequence found in the HIV-1 reverse transcriptase, and is thought to bridge the C-terminal domain of that protein to the penultimate domain. This peptide is known to be susceptible to mild proteolysis and thus is thought to be on the outside surface of the protein. The sequence is highly charged which should increase the solubility of any protein containing it. In fusing the IL3 and X molecules, multiple copies of the linker sequence of choice may be inserted between the two molecules. The present invention is, however, not limited by th form, size or number of linker sequenc s employed.

Another exemplary linker sequence useful in producing the fusion proteins of the present invention is Gly Ser Gly Ser Glu Asp Cys Glu Asp Ser Gly Ser Gly. In fact, the only requirement for the linker sequence is that it functionally does not interfere adversely with the folding of the individual components of the fusion molecule. Moreover, such linkers may be completely absent in a directly fused IL3-X or X-IL3 molecule.

Vectors for use in the construction of the fusion molecules and in the method of expression of the novel IL3-X or X-IL3 fusion proteins also form part of this invention. Vectors containing the IL3-X or X-IL3 DNA sequences which code for fusion proteins of the invention or vectors incorporating modified sequences as described herein are also embodiments of the present invention and useful in the production of IL3-X or X-IL3 proteins.

The vectors employed in the method also contain selected regulatory sequences in operative association with the DNA coding sequences of the invention and capable of directing the replication and expression thereof in selected host cells. The use of regulatory regions for controlling transcription of the fusion genes may allow for growing the host cells to high density with no or low levels of expression of the fusion gene, and then inducing expression by changing the environmental conditions, such as nutrient, temperature, and the like. Host cells transformed with such vectors for use in producing recombinant IL3-X or X-IL3 are also provided by the present invention.

The present invention also encompasses the novel fusion DNA sequences, free of association with DNA sequences encoding other primate proteins, and encoding IL3-X or X-IL3 fusion proteins. The DNA sequences may be fused in frame either directly or through a linker to bring the sequences within a preferred proximity to each other. Variations of DNA sequences encoding the IL3 and X peptide sequences are also included in the fusion molecule of the present invention as well as analogs or derivatives thereof. DNA sequences which

code for IL3 and X polypeptides but which differ in codon sequence from naturally occurring IL3 or X, due to the degeneracies of the genetic code or allelic variations (naturally occurring base changes in the species population which may or may not result in an amino acid change) are also encompassed by this invention. Variations in the DNA sequence of IL3 or X which are caused by point mutations or by induced modifications to enhance the activity, halflife or production of the fusion protein encoded thereby are also encompassed in the invention.

Modifications in the peptides or DNA sequences forming the fusion molecules of the present invention can be made by one skilled in the art using known techniques. Modifications of interest in the IL3 or X sequences may include the replacement, insertion or deletion of a selected amino acid residue in the coding sequences thereof, the insertion or destruction of a glycosylation site or other known peptide modifications. Such modifications may be made in the components of the fusion molecules to enhance the biological properties thereof. Mutagenic techniques for such replacement, insertion or deletion are well known to one skilled in the art. [See, e.g., United States patent 4,518,584.]

Other analogs and derivatives of the sequences of IL3 or X which would be expected to retain that molecules biological or physiological activity in whole or in part may also be easily made by one of skill in the art for use in the fusion molecule of the present invention given the disclosures herein. One such modification may be the attachment of polyethylene glycol to cysteine residues added to IL3 by replacement of existing residues or by insertion as described in PCT publication WO 90/12874, published November 1, 1990, or to X or in the linker peptide region. Such modifications are believed to be encompassed by this invention.

The present invention also provides a method for producing IL3-X or X-IL3 fusion molecules. The method involves (1) culturing in a culture medium a suitable cell or cell line,

which has been transform d with a DNA sequence coding on expression for an IL3-X or X-IL3 fusion molecule in operative association with an expression control sequence, under conditions permitting expression of the fusion protein, and (2) harvesting the fusion protein so produced from the culture media. Suitable preferred cells are bacterial cells. For example, the various strains of E. coli (e.g., HB101, MC1061 and strains used in the following examples) are well-known as host cells in the field of biotechnology. Various strains of B. subtilis, Pseudomonas, other bacilli and the like may also be employed in this method. Other suitable cells are mammalian cells, for example COS and CHO.

The selection of suitable cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. See, e.g., Gething and Sambrook, Nature, 293:620-625 (1981); Kaufman et al, Mol. Cell. Biol., 5(7):1750-1759 (1985) or Howley et al, U. S. Patent 4,419,446.

Yeast cells, fungal cells, or insect cells known to those skilled in the art may also be useful as host cells for expression of the fusion molecules of the present invention. See, e.g. Miller et al, Genetic Engineering, 8:277-298 (Plenum Press 1986) and references cited therein.

The harvesting of the fusion protein of the invention from a cell lysate or extract of the culture medium in any of the above described host cells may be performed by conventional protein isolation techniques.

Pharmaceutic compositions containing a therapeutically effective amount of the fusion proteins IL3-X or XIL-3, in admixtures with a pharmaceutically acceptable vehicle are included in this invention. These pharmaceutical compositions are suitable for the treatment of a number of pathological or disease states, particularly those characterized by a decreased level of either myeloid, erythroid, lymphoid, or megakaryocyte cells of the hematopoietic system or combinations thereof. In addition, the fusion proteins may be used for the preparation of pharmac utical compositions

suitable for activating mature myeloid and/or lymphoid cells. For example pharmaceutical compositions containing the IL3-IL11 fusion molecule may be useful in stimulating the production and/or development of megakaryocytes and platelets. Among conditions susceptible to treatment with the pharmaceutical compositions of the present invention is leukopenia, a reduction in the number of circulating leukocytes (white cells) in the peripheral blood. Leukopenia may be induced by exposure to certain viruses or to radiation. It is often a side effect of exposure to chemotherapeutic drugs. The pharmaceutical compositions of this invention may avoid undesirable side effects caused by presently available drugs. Additionally, the multidomain facet of the proteins of this invention may allow lower dosages of the pharmaceutical compositions to be used in comparison to use of pharmaceutical compositions containing the individual IL3 or X lymphokines alone.

Various immunodeficiencies or immune disorders may also be susceptible to treatment with the pharmaceutical compositions of the present invention. These pharmaceutical compositions, alone or in combination with other treatment regimens, may be useful in treating or correcting immunodeficiencies which are the result of viral infections, e.g., HIV, HTLVI or HTLVII, severe exposure to radiation, cancer therapy or the result of other medical treatment. Depending on the identification of X, the pharmaceutical compositions may be used to treat other blood cell deficiencies, including thrombocytopenia (platelet deficiency), or anemia (red cell deficiency). Other uses are in the treatment of patients recovering from bone marrow transplants.

Such pharmaceutical compositions comprise a therapeutically effective amount of the fusion protein IL3-X or X-IL3 of the present invention in admixture with a pharmaceutically acceptable carrier. This composition can be systematically administered parenterally. Alternatively, the composition may be administered intravenously. If desirable, the composition may b administered subcutaneously. Wh n systematically

administered, the therapeutic composition for us in this invention is in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such a pharmaceutically acceptable protein solution, having due regard to pH, isotonicity, stability and the like, is within the skill of the art.

The following examples describe the construction and production of illustrative fusion proteins of this invention.

Example 1 - Construction of Multidomain IL3 Molecules

To obtain an IL3-X fusion protein, two IL3 cDNA sequences were obtained according to the procedures described in PCT publication WO 88/00598, published January 28, 1988. The two cDNA sequences were fused together with a short piece of DNA. This DNA encoded a linker peptide designed to allow the two IL3 proteins to fold and act independently of one another.

The sequence of this linker peptide was based on a sequence found in the HIV-1 reverse transcriptase as described above. The sequence of the linker used in this fusion is as follows:

Gly Asp Ala Asn Arg Glu Thr Lys Leu Gly Lys Gly

C GGT GAT GCT AAC CGT GAA ACT AAG CTT GGT AAA GG
G CCA CTA CGA TTG GCA CTT TGA TTC GAA CCA TTT CCA T

The scheme used to fuse the IL3 cDNAs and the linker region employs conventional recombinant engineering techniques as described in Maniatis et al, "Molecular Cloning, A Laboratory Manual", Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1982). The construction of the fusions is outlined in Fig. I. Briefly described, the IL3 cDNA sequence, minus the sequence encoding its secretory leader, had the 5' and 3' ends that appear in the upper portion of Table A below. These IL3 sequences after digestion with XbaI and Mung Bean nuclease, or NdeI had the 5' and 3' ends that appear in the lower portion of Table A.

Table A

3' end of gene:	5' end of gene:
ATC TTC TAG A	CAT ATG GCT
TAG AAG ATC T	GTA TAC CGA
Ile Phe stop	Met Ala
--XbaI-	--NdeI-

3' end of gene:	5' end of gene:
ATC TT	T ATG GCT
TAG AA	AC CGA

The digested IL3 sequences were inserted into a plasmid, pALhIL3-781, designed for expression of heterologous proteins intracellularly in E. coli. This plasmid is described for illustration only. The techniques described to produce the described fusion molecules may employ other plasmids containing the same or different component sequences, restriction sites and the like. This exemplary plasmid is a modified form of pAL181 [ATCC Deposit #40134] containing an additional transcriptional terminator sequence. Plasmid pALhIL3-781, as depicted in Fig. I, is characterized by the complete cDNA sequence of mature IL3 fused in frame with an initiator methionine and an appropriately spaced ribosome binding site; the major leftward promoter from phage lambda to control and drive transcription of the IL3 cDNA; a sequence beyond the 3'-end of the IL3 sequence which causes transcription to terminate; and three unique restriction sites within the plasmid. One site 5' to the IL3 gene and encompassing the coding sequence for the initiator methionine is NdeI. Another site is incorporated within the translation termination sequence in the 3' end of the IL3 gene, XbaI. The remaining site is 5' to the transcription termination sequence, HindIII.

Two samples of the plasmid were prepared. One was cut with NdeI and HindIII and the small fragm nt containing the IL3

cDNA was purified (se right sid of Fig. I). The second was cut with XbaI, treated with Mung Bean exonuclease to remove the single-stranded DNA tails, and digested with HindIII. The larger fragment was isolated (see left side of Fig. I). The two isolated fragments were mixed with synthetic linker oligonucleotides of the sequence shown above and treated with T4-polynucleotide ligase.

The sequence of the junction region between the two IL3 sequences after ligation was as follows:

```
    Phe Gly Asp Ala Asn Arg Glu Thr Lys Leu Gly Lys Gly
TC TTC GGT GAT GCT AAC CGT GAA ACT AAG CTT GGT AAA GGT
AG AAG CCA CTA CGA TTG GCA CTT TGA TTC GAA CCA TTT CCA

Met
ATG G
TAC C
```

Plasmids in which the two IL3 coding sequences and one or more linker sequences had been fused together were selected by colonies that hybridize to linker sequences and the sequence of the fusion junctions verified. The presence of plasmids with more than one linker inserted between the IL3 sequences was unexpected. These multiple linker plasmids probably arose from a small amount of nuclease contamination in the DNA ligase or some carry-over of Mung Bean nuclease which removed the TA single-stranded tail of the linker duplex allowing it to be ligated to the blunt end of an adjacent linker duplex.

Once the plasmids were constructed, they were transformed into appropriate E. coli strains, such as W3110 (lambda PamcI857) [M. Rosenberg et al, Meth. Enzymol., 101:123-137 (1983) and Sambrook et al, cited above] for expression of the multidomain IL3. The two plasmids selected for this experiment contained two IL3 cDNA sequences separated by one and three linker sequences respectively. pALIL31-781 which contains one copy of the linker is illustrated in the lower portion of Fig. I. These fusion molecules produced proteins of 31 and 34 kilodaltons, respectively.

The proteins accumulated within the cells as insoluble inclusion bodies and were solubilized and refolded by standard m thods [see, .g., U. S. Patent No. 4,512,922]. When test d

in the CML assay described below in Example 9, the proteins demonstrat d IL3 activity equal to r greater than th activity of natural or recombinant IL3.

Example 2 - IL3/GMCSF Fusion Proteins

Another fusion protein was formed by fusing the IL3 cDNA sequence in frame with the DNA sequence encoding granulocyte-macrophage colony stimulating factor, GMCSF. The GMCSF cDNA sequence is described in European patent 188,479, published January 30, 1986. The two cDNA sequences were fused together with the short piece of linker DNA described in Example 1 by analogous techniques.

The scheme used to construct the DNA sequence which encodes the IL3-GMCSF fusion protein is provided schematically in Fig. II. The IL3 cDNA was inserted into the expression plasmid, pAL181, modified as described in Example 1. The GMCSF cDNA, minus the sequence encoding its secretory leader, was inserted into an unmodified version of pAL181, resulting in plasmid pALC-186. The IL3 plasmid, pALhIL3-781, was digested with XbaI and then treated with Mung Bean exonuclease to remove the single-stranded DNA tails. The plasmid was then digested with AvaI and the small resultant fragment carrying the IL3 coding sequence was isolated. The GMCSF expression plasmid, pALC-186, was digested with NdeI and AvaI, and the large fragment which carried the GMCSF gene was isolated. The two fragments were mixed with synthetic oligonucleotides encoding the linker peptide described in Example 1 and treated with T4-polynucleotide ligase. Plasmids were selected in which the IL3 cDNA, linker oligonucleotide, and the GMCSF cDNA sequences were fused contiguously to form a gene which encodes a protein with IL3 as its N-terminal domain and GMCSF as its C-terminal domain.

The plasmid carrying the gene for the IL3-GMCSF fusion protein, pALIL3-GM181, was transformed into an appropriate E. coli hcst strain as described in Example 1 for expression of the fusion protein. When expressed, the fusion protein accumulates within the c lls as insoluble inclusion bodies,

which are solubilized and refold d by th standard methods d scribed above. Activity of this fusion protein is t sted in the [CML] assay described in Example 9.

Example 3 - IL3/GCSF Fusion Proteins

Another exemplary fusion protein of formula IL3-X was formed by fusing the IL3 cDNA sequence in frame with the DNA sequence encoding granulocyte-colony stimulating factor, GCSF. GCSF cDNA for this fusion was obtained from Genetics Institute, Inc. and had the sequence in PCT publication WO 87/01132, published February 26, 1987. This fusion molecule was constructed in a manner analogous to that described for IL3-IL3. Specifically, the fusion was mediated by the linker DNA sequence described in Example 1.

The scheme used to construct the DNA sequence which encodes an IL3-GCSF fusion protein is provided schematically in Fig. III. The IL3 and the GCSF cDNAs, minus these leader sequences, were inserted into the expression plasmids, pAL181, modified as described in Example 1. The IL3 expression plasmid pALhIL3-781 was cut with XbaI, treated with Mung Bean exonuclease to remove the single-stranded DNA tails, and digested with HindIII. The larger fragment was isolated. The GCSF expression plasmid pALG$_a$-781 was cut with NdeI and HindIII and the small fragment containing the GCSF gene was purified. The two isolated fragments were mixed with synthetic oligonucleotides encoding the same linker peptide used in Example 1 and treated with T4-polynucleotide ligase. Plasmids were selected in which the IL3 cDNA, linker oligonucleotide, and GCSF cDNA sequences were fused contiguously to form a gene which encodes a protein with IL3 as its N-terminal domain and GCSF as its C-terminal domain.

The plasmid carrying the gene for the IL3-GCSF fusion protein, pALIL3G-781, was transformed into an appropriate E. coli host strain as described in Example 1 for expression of the fusion protein.

When expressed, the fusion protein accumulated within the c lls as insolubl inclusion bodies and was solubiliz d and

refolded by the standard methods described above. The resulting protein had IL3 activity and GCSF activity in the MO7E, 32D and DA2 proliferation assays described below in Example 9.

Example 4 - GCSF/IL3 Fusion Proteins

An exemplary fusion protein of formula X-IL3 was formed by fusing the IL3 cDNA sequence in frame with, and 3' to, the DNA sequence encoding granulocyte-colony stimulating factor, GCSF. This fusion molecule was constructed in a manner analogous to that described for IL3/GCSF. Specifically, the fusion was mediated by the linker DNA sequence described in Example 1.

The scheme used to construct the DNA sequence which encodes the GCSF-IL3 fusion protein is provided schematically in Fig. IV. The IL3 and the GCSF cDNAs were inserted into the modified expression plasmids pAL181 described in Example 1. The GCSF cDNA had a StyI site at its 3' end and another one internal to the coding sequence of the gene. The internal site was changed by site-directed mutagenesis, leaving the protein sequence unaltered. The resultant plasmid, $pALG_b$-781, was cut with StyI, treated with Mung Bean exonuclease to remove the single-strand DNA tails, and digested with HindIII. The larger fragment was isolated. The IL3 expression plasmid, pALhIL3-781, was cut with NdeI and HindIII and the small fragment containing the IL3 gene was purified. The two isolated fragments were mixed with synthetic oligonucleotides encoding the same linker peptide used in Example 1 and treated with T4-polynucleotide ligase. Plasmids were selected in which the GCSF, linker oligonucleotide, and IL3 cDNA sequences were fused contiguously to form a gene which encodes a protein with GCSF as its N-terminal domain and IL3 as its C-terminal domain.

The plasmid carrying the gene for the GCSF-IL3 fusion protein, pALGIL3-781, was transformed into an appropriate E. coli host strain as described in Example 1 for expression of the fusion protein.

When expressed, the fusion protein accumulated within the

cells as insoluble inclusion bodies and was solubilized and refold d by the standard methods described above. The fusion protein had both GCSF and IL3 activity in the in vitro cell stimulation assays MO7E, 32D, and DA2 proliferation assays described in Example 9.

Example 5 - IL3/IL11 Fusion Protein

Another exemplary fusion protein of formula IL3-X was formed by fusing the IL3 cDNA sequence in frame with the DNA sequence encoding the mature form of interleukin 11 [IL11]. The sequence of IL11 and methods for obtaining it are described in detail in PCT publication WO 91/07495, published May 30, 1991. The IL11 cDNA was constructed from synthetic oligonucleotides to encode a protein which has the same primary amino acid sequence as found in human IL11, but using codons more compatible with bacterial expression than the native cDNA. The sequence of this modified IL11 gene is provided below in Table B. The gene fusion was constructed in a manner analogous to that described in Example 3. Specifically, the fusion was mediated by the linker DNA sequence described in Example 1.

TABLE B

ATGCCAGGTC CACCACCAGG TCCACCTCGA GTTTCCCCAG ACCCGCGCGC
TGAACTGGAC AGCACAGTAC TGCTGACCCG CTCTCTGCTG GCAGACACTC
GCCAGCTGGC TGCACAGCTG CGCGACAAAT TCCCGGCTGA CGGTGACCAC
AACCTGGATT CCCTGCCGAC CCTGGCTATG AGCGCAGGTG CACTGGGAGC
TCTGCAACTG CCAGGTGTAC TGACTCGCCT GCGTGCAGAC CTGCTGTCCT
ACCTGCGCCA CGTTCAGTGG CTGCGGCGCG CAGGTGGCTC TTCCCTGAAA
ACCCTGGAAC CGGAGCTGGG CACCCTGCAA GCTCGCCTGG ACCGCCTGCT
GCGCCGCCTG CAGCTGCTGA TGTCCCGCCT GGCTCTGCCG CAGCCACCAC
CGGACCCACC AGCACCGCCG CTGGCTCCAC CATCCTCTGC TTGGGGTGGT
ATCCGCGCAG CTCACGCTAT CCTGGGTGGT CTGCACCTGA CTCTGGACTG
GGCTGTTCGC GGTCTGCTGC TGCTGAAAAC TCGCCTGTAA TAG

The scheme used to construct the DNA sequence which encodes an IL3-IL11 fusion protein is provid d schematically in Fig.

V. The IL3 expression plasmid, pALhIL3-781, was treated as described in Example 3. The IL11 sequence was synthesized by ligating 70-90 bp oligonucleotides together in about 150 bp pieces and inserting them sequentially into expression plasmid, pAL181, to build up the entire gene. Once the entire IL11 coding sequence had been constructed, the plasmid, called pALIL11-781, was capable of directing synthesis of the IL11 protein. The expression plasmid was digested with NdeI and HindIII and the small fragment containing the IL11 gene was purified. The IL11 cDNA fragment and the cleaved IL3 expression vector prepared as described in Example 1, were mixed with synthetic oligonucleotides encoding the linker peptide described in Example 1 and treated with T4-polynucleotide ligase. Plasmids were selected in which the IL3 cDNA, linker oligonucleotide, and IL11 gene sequences were fused contiguously to form a new gene which encodes a protein with IL3 as its N-terminal domain and IL11 as its C-terminal domain.

The plasmid carrying the gene for the IL3/IL11 fusion protein, pALIL311-781, was transformed into an appropriate E. coli host strain and the fusion protein was expressed and harvested as described in Example 1. The harvested fusion protein had activity in the T10 assay and MO7E assays described in Example 9.

Example 6 - IL3/IL9 Fusion Protein

Another exemplary fusion protein of formula IL3-X is formed by fusing the IL3 cDNA sequence in frame with the DNA sequence encoding the mature form of interleukin 9 [IL9]. The sequence of IL9 and methods for obtaining it are described in detail in PCT publication WO 90/14432, published November 29, 1990.

The IL9 cDNA described in the disclosure above has a site for cleavage with BamHI at nucleotide 94 and a site for cleavage with HindIII at nucleotide 490. This 396 base pair fragment is isolated from the original cDNA clone. This IL9 cDNA fragment and a cleaved IL3 expression vector prepared as described in Example 1 are mixed with oligonucleotides

encoding the linker sequence and the first seven codons of the mature IL9 protein. Th sequence of this linker is as follows:

CGGT GAT GCT AAC CGT GAA ACT AAG CTT GGT AAA GGT TGT CCA ACT CTG GCT GG
GCCA CTA CGA TTG GCA CTT TGA TTC GAA CCA TTT CCA ACA GGT TGA GAC CGA CCTAG

The mixture is treated with T4-polynucleotide ligase. Plasmids are selected in which the IL3 cDNA, linker oligonucleotides, and IL9 cDNA sequences are fused contiguously to form a new gene which encodes a protein with IL3 as its N-terminal domain and IL9 as its C-terminal domain.

The plasmid carrying the gene for the IL3/IL9 fusion protein is transformed into an appropriate E. coli host strain as described in Example 1 for expression of the fusion protein. Activity of this fusion protein is tested in the MO7E assay described in Example 9.

Example 7 - IL3/IL6 Fusion Protein

Another exemplary fusion protein of formula IL3-X is formed by fusing the IL3 cDNA sequence in frame with the DNA sequence encoding the mature form of interleukin 6 [IL6]. The sequence of IL6 and methods for obtaining it are described in detail in PCT publication WO 88/00206, published January 14, 1988.

The IL6 cDNA contained within plasmid pAL309C-781 described in the disclosure above is cleaved with restriction endonucleases NdeI and HindIII. The small IL6 encoding fragment is purified. This IL6 cDNA fragment and a cleaved IL3 expression vector prepared as described in Example 1 are mixed with synthetic oligonucleotide encoding the linker peptide described in Example 1 and treated with T4-polynucleotide ligase. Plasmids are selected in which the IL3 cDNA sequences are fused contiguously to form a new gene which encodes a protein with IL3 as its N-terminal domain and IL6 as its C-terminal domain.

The plasmid carrying the gene for the IL3/IL6 fusion protein is transformed into an appropriate E. coli host strain as described in Example 1 for expression of the fusion protein. Activity of this fusion protein is tested in the MO7E and T10 assays described in Example 9.

Example 8 - Expression of Recombinant IL3-X Fusion Proteins

To express the fusion proteins of the exampl s, the DNAs in the plasmids described above encoding the fusion proteins are transferred into appropriate expression vectors, of which numerous types are known in the art for mammalian, insect, yeast, fungal and bacterial expression, by standard molecular biology techniques.

a. Bacterial Expression Systems

One skilled in the art can manipulate the sequences encoding the IL3-X and X-IL3 proteins by eliminating any mammalian regulatory sequences flanking the coding sequences and inserting bacterial regulatory sequences to create bacterial vectors for intracellular or extracellular expression of the fusion proteins of the invention by bacterial cells. The DNA encoding the fusion proteins may be further modified to contain different codons to optimize bacterial expression as is known in the art. The sequences encoding the fusion proteins may be operatively linked in-frame to nucleotide sequences encoding a secretory leader polypeptide permitting bacterial expression, secretion and processing of the fusion proteins by methods known in the art. Alternatively the IL3-X or X-IL3 fusions may be constructed for intracellular expression and the protein isolated, mixed and refolded by procedures well known in the art. The fusion protein expressed through either route in bacterial host cells may then be recovered, purified, and/or characterized with respect to physicochemical, biochemical and/or clinical parameters, all by known methods.

b. Mammalian Cell Expression

To obtain expression of the fusion protein a vector for mammalian cells, pXM, and the general procedures described in Y. C. Yang et al, Cell, 47:3-10 (1986) may be used. See, also, Kaufman, Proc. Natl. Acad. Sci. USA, 82:689-693 (1985), Kaufman et al, J. Mol. Biol., 159:511-521 (1982); and Kaufman, Proc. Natl. Acad. Sci., USA, 82:689-693 (1985) for descriptions of vector construction techniques and vector components us ful in the practic of this inv ntion.

One skilled in the art can also c nstruct other mammalian expression vectors comparable to the pXM vector by, e.g., inserting the DNA sequences of the fusion proteins from the respective plasmids with appropriate enzymes and employing well-known recombinant genetic engineering techniques and other known vectors.

For stable integration of the vector DNAs, and for subsequent amplification of the integrated vector DNAs, CHO cells may be employed using conventional methods. The transformation of these vectors with IL3-X or X-IL3 into appropriate host cells can result in expression of the fusion proteins. The resulting cell lines can be further amplified by appropriate drug selection, resulting cell lines recloned and the level of expression assessed using the appropriate assay for the components of the IL3-X fusion protein. This approach is particularly useful when X is desirably glycosylated, e.g. erythropoietin.

c. Insect or Yeast Cell Expression

Similar manipulations can be performed for the construction of an insect vector for expression of these fusion proteins in insect cells [See, e.g., procedures described in European patent 155,476, published September 25, 1985].

Similarly yeast vectors are constructed employing yeast regulatory sequences to express the fusion protein in yeast cells to yield intracellularly expressed or secreted extracellular active fusion protein. [See, e.g., procedures described in PCT publication WO 86/00639, published January 30, 1986, and European patent EP 123,289, published October 31, 1984.] Fungal vectors may also be employed in the expression of these fusion molecules.

Example 9 - Biological Activities of IL3-X

In all proliferation assays described, the basic format is as follows:

The samples and standards are diluted in assay medium in U-bottomed microtiter plates in a final volume of 100μl/well. Five-fold serial dilutions are appropriate in most cases. The targ t c lls are harv sted from activ ly growing cultur s,

centrifuged, washed and resuspended in assay medium at concentrations which have been optimized for each assay; 100µl of cell suspension is added to each well so that the final volume is 200µl/well. The plates are incubated at 37°C in a fully humidified incubator and pulsed with 0.5uCi [^{3}H]-thymidine/well as described in each assay below. Proliferation is measured by the incorporation of [^{3}H]-thymidine into the cells. Bioactivity is measured in half maximal dilution units where a final concentration of 1 unit per ml of IL3/X fusion results in 50% incorporation of [^{3}H]-thymidine in the assay.

a. CML Assay

The CML assay for stimulating proliferation of leukemic blast cells was performed essentially according to procedures described in Blood, 63(4):904-111 (1984). A stock of cells was obtained from a frozen bag of peripheral blood from a CML patient in stable phase. The bag was thawed and refrozen in 500 aliquots of 1.7×10^7 cells/vial. One day prior to setting up the assay a vial of cells is thawed and the cells are washed twice with 10ml of RPMI + 5% heat-inactivated Human AB serum (HiHAB). The cells are resuspended in 10ml of the same medium and incubated overnight in 5% CO_2 at 37°C. The following day the cells are removed from culture, ficolled, washed and resuspended in assay medium.

The assay is performed in RPMI + 10% heat-inactivated fetal calf serum (HiFCS), 2mM glutamine (GLN) and P/S (100U/ml penicillin, 100ug/ml streptomycin). The seeding density for the cells is 2×10^4 cells/well. The plates are incubated for 48 hours in 5% CO_2 and pulsed with [^{3}H]-thymidine for the final 6 hours of the assay. The CML cells respond to both hGMCSF and hIL3. Therefore, to quantitate the activity of each cytokine separately in the fusion protein of example 2, it is necessary to include neutralizing antibody to either hGMCSF or hIL3.

b. M07E Assay

The M07E cell line was derived from the peripheral blood of an infant with acute megakaryoblastic leukemia. Growth of

M07E cells is dependent on the presence of human GMCSF, human IL3 or human IL4.

M07E cells are maintained in DME plus 10% heat-inactivated FCS, glutamine and P/S supplemented with 8 units per milliliter recombinant human IL-3. The assay is performed in same medium without addition of human IL-3. The seeding density of the cells is 10^4 cells per well. The plates are incubated for 3 days in 10% CO_2 and pulsed for the final 4 hours with [^{3}H]-thymidine.

As described in the CML assay, the M07E cells respond to both hGMCSF and hIL3, requiring the use of neutralizing antibodies.

Based on the incorporation of [^{3}H]-thymidine, the IL3-containing fusion proteins of all IL-3/X fusion examples are active in stimulating the proliferation of leukemic blast cells in this CML assay.

c. TF-1 Assay

The TF-1 cell line was derived from the bone marrow of a patient with erythroleukemia [T. Kitamura, University of Tokyo]. The cells are grown in RPMI plus 10% heat-inactivated FCS, glutamine and P/S, supplemented with 100 units per ml recombinant human GMCSF. The assay is performed in the same medium without addition of hGMCSF. The seeding density of the cells is 7.5×10^3 cells per well. The plates are incubated for 3 days in 5% CO_2 and pulsed for the final 4 hours with ^{3}H-thymidine. As described in the CML assay, the TF-1 cells respond to both hGMCSF and hIL3 requiring the use of neutralizing antibodies.

Based on the thymidine uptake measurement, the IL3/X fusion proteins are active in this assay in stimulating the proliferation of these erythroleukemia cells.

d. 32D Proliferation Assay

32D is a murine IL3-dependent cell line grown in RPMI with 10% heat-inactivated FCS, glutamine, and P/S with 20% WeHi 3B conditioned medium as a murine IL3 source. These cells proliferate in the presence of GCSF.

The assay is performed in RPMI, containing 5% heat-

inactivated FCS, glutamin and P/S. The seeding density is 2 x 10^4 cells per well. The plates are incubated for 24 hours in 5% CO_2 and pulsed for the final 4 hours with [^{3}H]-thymidine.

e. DA2 Proliferation Assay

DA2 is a LIF dependent murine cell line which grows equally well in murine IL-3. The cells are maintained in RPMI with 5% heat-inactivated FCS, glutamine, P/S and 500 units/ml recombinant human LIF.

The DA-2 assay is performed in the same medium as the 32D assay. The seeding density of the cells is 7.5 x 10^3 cells per well. The plates are incubated for 3 days and pulsed for the final 4 hours with [^{3}H]-thymidine.

f. T10 Proliferation Assay

This assay was described in detail in PCT publication WO 91/07495, published May 30, 1991.

T10 cells are a subpopulation of the IL-6 dependent murine plasmacytoma cell line T1165 [R. P. Nordan et al, Science, 233:566 (1986); and obtained from Dr. Nordan, National Institutes of Health] that were selected for growth in IL-11. The T10 cell line responds well to either IL6 or IL11 but the response to IL11 is much greater than that of the original T1165 cell line. The cells are maintained in RPMI containing 10% heat-inactivated FCS, glutamine, P/S, 5 x 10^{-5} M beta mercaptoethanol (Sigma Chemical Co., St. Louis, MO), and supplemented with 20 U/ml rhuIL11. The assay is performed in the same medium without IL11. The seeding density is 7.5 x 10^3 cells per well. The plates are incubated for 3 days and pulsed for the final 3 hours with [^{3}]-thymidine.

E. coli cell supernatants from the transfection of the pALIL311-781 described in Example 5 were assayed for activity in accordance with the above. Th IL3-IL11 fusion protein

reveal d IL11 activity in this assay.

g. Human Plasma Clot meg-CSF Assay

The fusion molecule IL3-IL11 of this invention was also tested for human megakaryocyte colony formation activity in the plasma clot meg-CSF assay described in E. Mazur et al., Blood 57:277-286 (1981) with modifications. Non-adherent peripheral blood cells were isolated from Leukopacs and frozen in aliquots. The test sample was mixed with platelet-poor human AB plasma and 1.25 x 10^5 cells in 24-well plates and allowed to clot by the addition of calcium. After a 12 day incubation, megakaryocytes were identified using a monoclonal antibody directed against platelet glycoproteins IIb/IIIa and a horseradish peroxidase/ anti-peroxidase chromogenic detection system. Recombinant human IL-3 [Genetics Institute, Inc.] was used as a positive control, producing 12-30 megakaryocyte colonies per clot with approximately 60% pure and 40% mixed megakaryocyte colonies. Aplastic dog serum was also used as a positive control, which produced between 5-10 megakaryocyte colonies per clot, of which approximately 50% were pure megakaryocyte colonies containing less than 10 cells, and 50% were mixed megakaryocyte colonies containing more than 40 megakaryocytes. The negative control was Alpha Medium, which produced 0-1 megakaryocyte colonies per clot.

The IL3-IL11 fusion protein was compared with optimal concentrations of IL3 protein and IL11 protein. Two series of experiments were performed. In the first series, IL11 concentration was maintained constant at its optimal concentration of 10 U/ml and IL3 conc ntration was vari d from

0.2 - 200 U/ml. In the second series, IL3 concentration was maintained constant at its optimal concentration of 1 U/ml and IL11 concentration was varied from 0.2 - 200 U/ml. The optimal concentrations were determined by carrying out the assay with IL3 alone and with IL11 alone at concentrations ranging from 0.2 - 200 U/ml. The concentration resulting in the highest number of meg colonies/clot produced was then chosen as the optimal concentration for the comparison experiments.

The results are shown below:

[IL3] U/ml	[IL11] U/ml	Meg Colonies/Clot
0.2	10	3
1.0	10	12
5.0	10	12
10.0	10	24
50.0	10	24
100.0	10	28
200.0	10	27
1	0.2	16
1	1.0	32
1	5.0	14
1	10.0	29
1	50.0	19
1	100.0	18
1	200.0	12

The above results were compared to the following results obtained with varying concentrations of the IL3-IL11 fusion protein, in a ratio of 5 units IL11 to 1 Unit IL3. This ratio was determined to mimic the relative specific activities of the two fusion partners, as determined in the T10 and MO7E cell proliferation assays.

[IL3-IL11 Fusion Protein] U/ml	Meg Colonies/Clot
0.2/1.0	14
1.0/5.0	26
5.0/25	20
25.0/125	15
125/625	26

As can be seen, the IL3-IL11 fusion protein was at least as active in stimulating megakaryocyte colony formation as the optimal concentrations of IL3 and IL11 added together.

Numerous modifications and variations in practice of this invention are expected to occur to those skilled in the art.

CLAIMS

1. A fusion protein of the formula IL3-X or X-IL3 substantially free from association other proteinaceous materials, wherein X is a lymphokine fused to IL3 and selected from the group consisting of IL3, IL6, IL7, IL9, IL11, erythropoietin and GCSF.

2. The protein according to claim 1 wherein said lymphokine is IL11.

3. The protein according to claim 1 wherein said lymphokine is IL3.

4. The protein according to claim 1 wherein said lymphokine is erythropoietin.

5. The protein according to claim 1 wherein said lymphokine is GCSF.

6. The protein according to claim 1 wherein said lymphokine is IL9.

7. The protein according to claim 1 wherein said lymphokine is IL6.

8. The protein according to claims 1-6 wherein X is fused to IL3 through a peptide linker sequence.

9. A DNA sequence coding for a fusion protein of claim 1.

10. A plasmid vector comprising the DNA sequence of claim 9, in operative association with an expression control sequence capable of directing expression of the DNA sequence of claim 9 in a host cell.

11. A suitable host cell transformed with a plasmid vector of

claim 10.

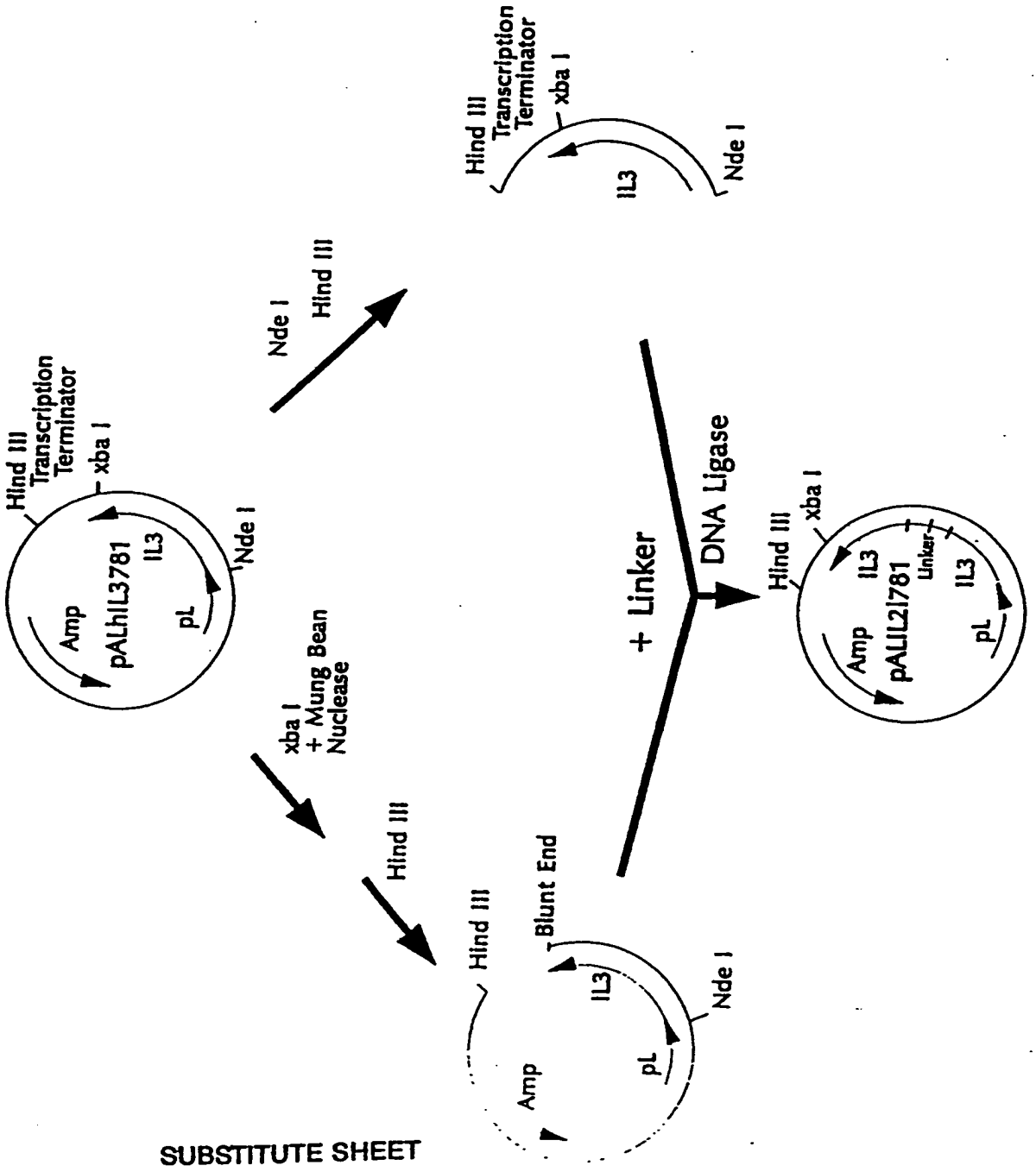
12. A process for producing a fusion protein IL3-X or X-IL3 comprising (1) culturing a suitable host cell of claim 11 in a culture medium under conditions permitting expression of the protein and (2) harvesting the fusion protein from the culture media.

13. A protein produced by the process of claim 12.

14. A pharmaceutical composition comprising a therapeutically effective amount of a fusion protein of claims 1-8 in a pharmaceutically acceptable vehicle.

15. Use of the composition of claim 14 in the preparation of a pharmaceutical composition for the treatment of leukopenia.

Figure 1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Hind III
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Figure 2

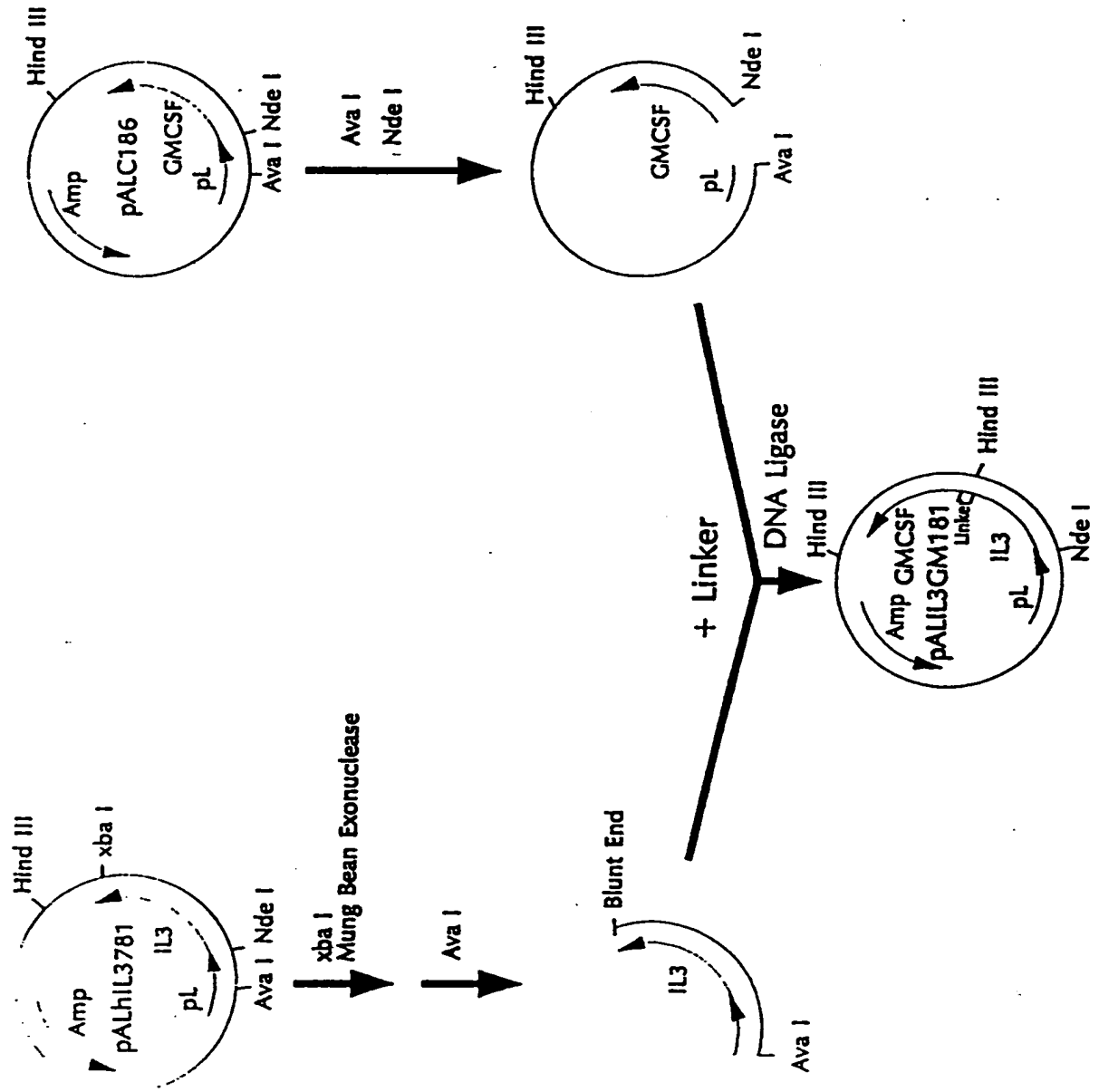

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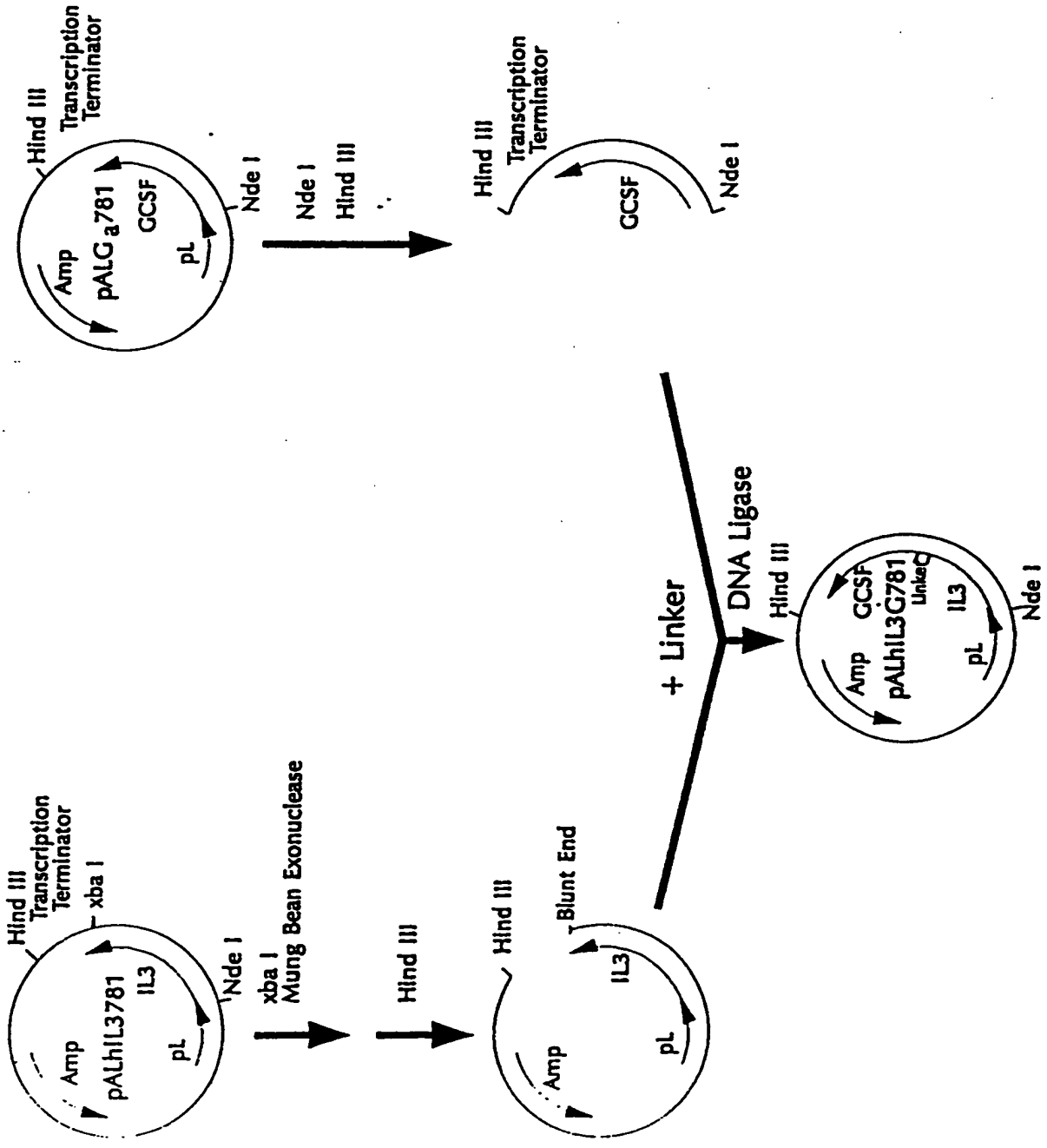
Figure 3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Figur 4

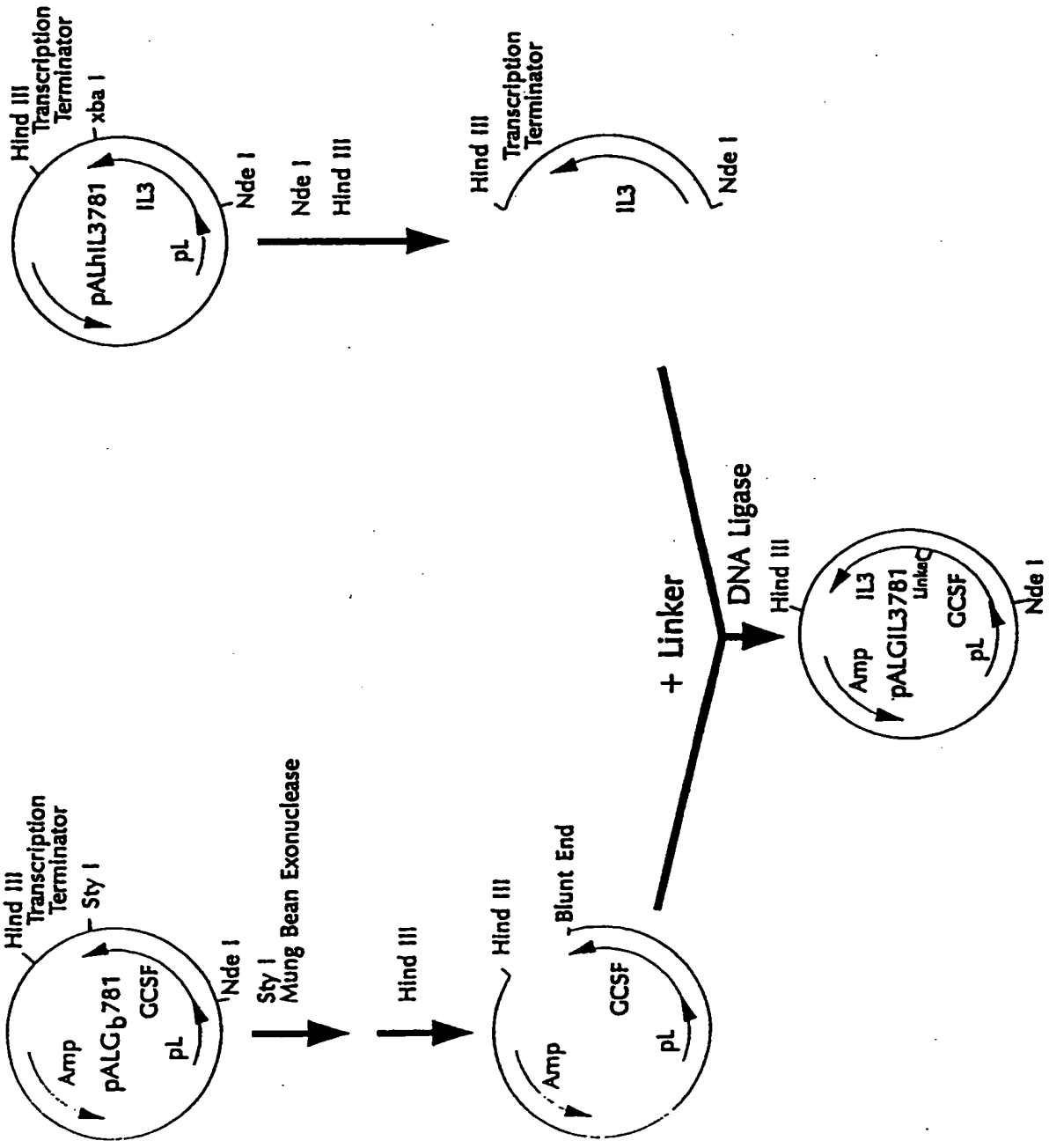
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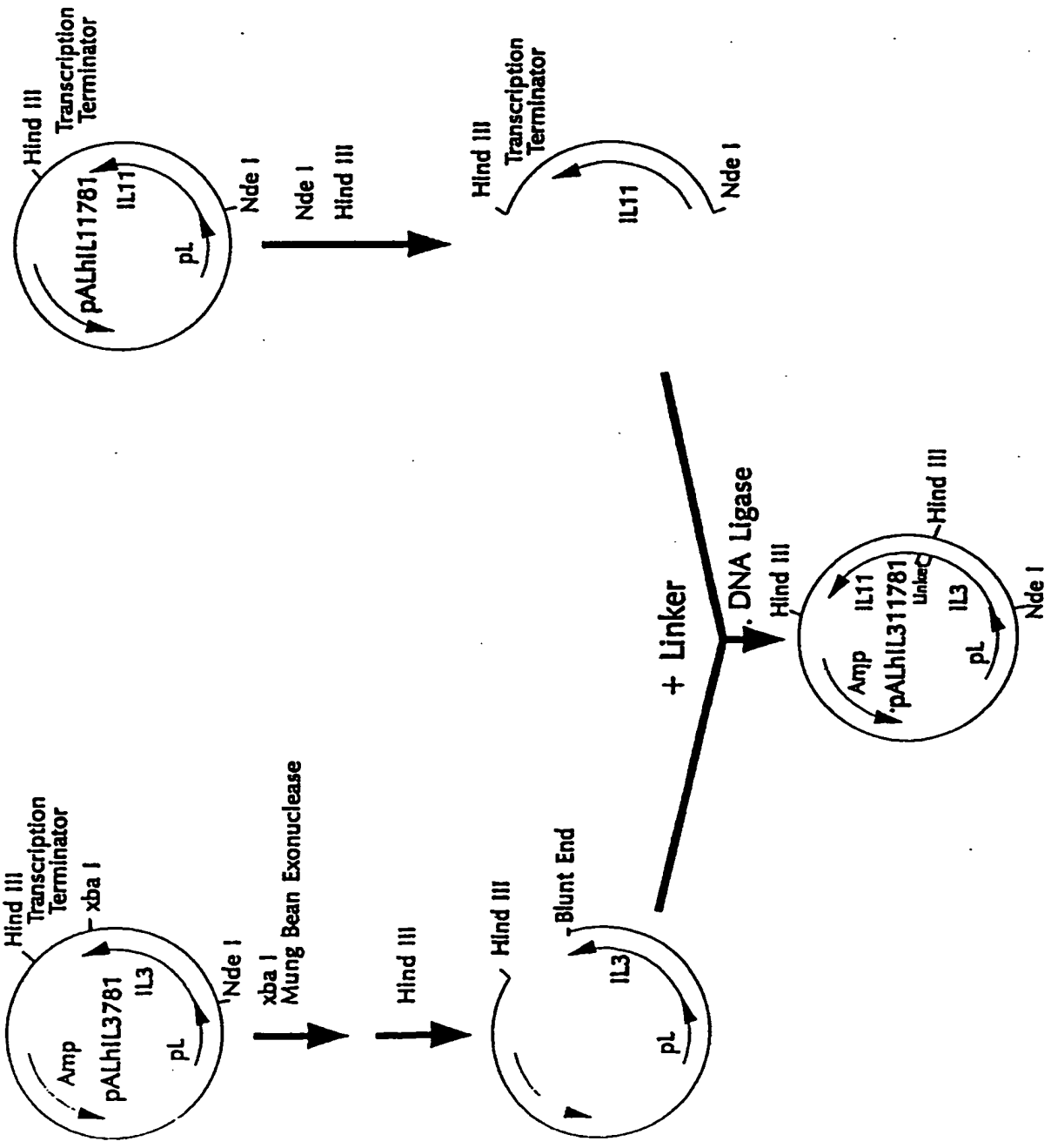
Figure 5



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